THE POTENTIAL ROLE OF A NEW LIM FACTOR, LMO4, IN BREAST CANCER

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Many properties of breast cancer cells, including increased proliferation and invasion, are common to epithelial cells of the developing mammary gland, suggesting that understanding of developmental control in normal mammary glands may provide important insights into the biology of breast cancer. This notion is supported by work in many organ systems, demonstrating that subversion of developmental control genes plays roles in carcinogenesis. LIM domain factors and associated co-regulators are important developmental regulators involved in pattern formation and organogenesis in a wide spectrum of organisms, including mammals. We isolated a LIM only factor, LMO-4, which is highly expressed in epithelial cells, including mammary epithelium. Interestingly, LMO factors are known to be oncogenic in lymphocytes where their overexpression causes acute lymphocytic leukemia.

We have studied expression of LMO-4 in mammary glands of mice and found that it is most highly expressed in proliferating mammary epithelial cells during pregnancy, suggesting that the LMO-4 gene may play a role in proliferation. Since LMOs do not bind to DNA it is likely that they regulate transcription by interacting with DNA-binding proteins and transcriptional co-regulators. To search for such factors, we have screened a human breast cDNA library with LMO-4 as bait in the yeast two hybrid system and found several potential interacting partners, including DNA-binding proteins, Clim/Nli/Ldb co-regulators and a splicing factor previously shown to be amplified in breast cancer cell lines. To test the role of LMO-4 in mammary gland biology, we have generated three lines of transgenic mice expressing under control of the MMTV promoter a) wild-type LMO-4, b) LMO-4 fused to the VP-16 transactivation domain and c) LMO-4 fused to the engrailed repression domain. Whole mount mammary gland analyses of these transgenic mice is in progress and preliminary results will be presented. Analyses of the EST databases indicate that LMO-4 is highly expressed in mammary carcinomas and we are in the process of evaluating its expression in breast cancer.

We conclude that LMO-4 may be an important regulator of mammary epithelial cells and propose a hypothesis that its high level expression in mammary tumors may play a role in mammary carcinogenesis.

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INVESTIGATING THE ROLE OF LAF-4 IN AXILLARY NODE-NEGATIVE BREAST CANCER

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LAF-4 is a gene that we have identified as being transcriptionally perturbed in breast cancer and thus may play an important role in breast cancer development. LAF-4 has previously been shown to have transactivation potential and is highly homologous to AF-4, a gene implicated in acute lymphoblastic leukemia. In agreement with previous finding that LAF-4 expression is restricted to cells of the lymphoid lineage, we did not detect LAF-4 mRNA in normal mammary epithelial cell lines. In contrast, the transcript was detected in some breast cancer cell lines, suggesting that LAF-4 expression may become aberrantly activated during mammary tumorigenesis. To determine whether expression of LAF-4 is perturbed in primary breast tumors, quantitative RT-PCR analysis of LAF-4 mRNA level was evaluated in axillary node negative (ANN) tumor tissue in comparison to corresponding normal mammary tissue. It was found to be up-regulated in 2/7 cases. Analysis of LAF-4 expression in a large cohort of ANN breast tumors showed that for the majority of tumors, the distribution of LAF-4 mRNA level was comparable to that found in normal mammary tissue. However, in 14/56 ANN tumors, the level of LAF-4 mRNA was significantly higher than the highest level found in the normal tissues. In addition, i*n situ* hybridization studies indicated that LAF-4 expression was specific to breast tumor cells. These results suggest that the aberrant activation of LAF-4 expression may contribute to the development of some breast cancers.

In investigating the mechanism underlying LAF-4 transcriptional activation in some breast tumors, we examined the status of DNA methylation of the LAF-4 promoter region. We hypothesize that the loss of DNA methylation may explain the presence of LAF-4 mRNA in some tumors. Breast tumor genomic DNA was examined using methylation specific PCR. We found that the LAF-4 promoter was not methylated irrespective of LAF-4 expression level. Methylation does not appear to be responsible for the aberrant activation of LAF-4, indicating the involvement of alternative mechanisms.

Our preliminary results demonstrate potential involvement in breast cancer pathogenesis of LAF-4. We are characterizing the functions of this gene to clarify its role in breast cancer. If this gene proves to have a significant role in breast cancer, it will be useful to evaluate its prognostic potential in ANN breast cancer in long-term studies. A clear understanding of the timing of transcriptional deregulation will allow the design of targeted prevention strategies and offers the potential of substantially reducing the number of new breast cancer cases in the future.

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ABERRANT PROMOTER METHYLATION OF THE TRANSCRIPTION FACTOR GENES PAX 5 ALPHA AND BETA IN BREAST CANCER

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Breast cancer is the second leading cause of cancer-related death in the United States and is increasing each year. Mortality from this disease could be reduced greatly through an improved understanding of the molecular alterations that contribute to disease initiation and progression. A common mechanism in many sporadic cancers, aberrant promoter hypermethylation, is an epigenetic event involving the methylation of normally unmethylated cytosines within the promoter region of genes. This change in methylation pattern leads to transcriptional silencing and serves as an alternative to coding region mutation. The purpose of the current study was to identify novel genes inactivated by promoter hypermethylation in breast cancer. A PCR-based technique of methylated CpG island amplification (MCA) followed by representational difference analysis (RDA) was used to identify genes methylated in breast cancer. Two of the CpG islands identified mapped to the 5' UTR region of the Pax 5 alpha and beta genes. These genes, located on chromosome 9p13, are transcribed from two distinct promoters and result in the formation of two alternative first exons that are subsequently spliced to the common exons 2-10. The resulting splice variants encode two distinct transcription factors important in cell differentiation and embryonic development. Examination of the methylation status of each gene using methylation-specific PCR (MSP) revealed that both genes are methylated in approximately 65% of breast tumors. No methylation was detected in peripheral blood lymphocytes. Analysis of methylated cell lines and tumors by combined bisulfite restriction analysis (COBRA) and bisulfite sequencing revealed dense methylation patterns within each 5'CpG island, strongly correlating with transcriptional silencing. Expression in cell lines with dense methylation of either the Pax 5 α or β promoter region was restored following treatment with the demethylating agent 5-Aza-dC. Recent studies have demonstrated the importance of Pax 5 gene alterations in human cancer. Our results are the first to identify aberrant promoter methylation as a mechanism for dysregulation of these genes.

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OVEREXPRESSION OF ERBB2 TRANSCRIPTIONALLY REGULATES THE VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) GENE THROUGH A NOVEL PROMOTER ELEMENT

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Breast cancer is the leading cause of death in American women between the ages of 20 and 59, and tumor vascularization is a critical step in cancer progression. To effectively treat breast cancer, it is important to understand the mechanisms by which cancer cells recruit and maintain blood vessels. One-third of all invasive breast tumors express high levels of ErbB2 (an epidermal growth factor receptor family member), which is correlated with poor patient prognosis. ErbB2 overexpression increases cell proliferation in vivo and promotes blood vessel recruitment to tumors. We are testing the hypothesis that the mechanism for this is upregulation of the pro-angiogenic factor VEGF. VEGF is a critical component of pathological tissue vascularization and is required for normal vascular development. Our lab and others have demonstrated that ErbB2 activity induces VEGF transcription. The current study is designed to identify the promoter element(s) through which ErbB2 overexpression regulates VEGF expression.

To determine the requirements for VEGF transcriptional regulation, we are conducting a structure-function analysis of the VEGF promoter in cultured mammary cells that overexpress ErbB2. We have generated a series of deletions in the VEGF promoter and are quantitating reporter gene expression to measure VEGF promoter activity.

Current models suggest that ErbB2 upregulates VEGF by increasing hypoxia inducible factor 1 (HIF1) protein levels. HIF1 binds a known VEGF promoter element. We have identified a novel ErbB2 overexpression-sensitive enhancer in the VEGF promoter. We conclude that ErbB2 mediated upregulation of VEGF involves at least two distinct promoter elements. Elucidation of the mechanisms by which ErbB2-overexpressing tumors can recruit blood vessels will also allow for a better understanding of the progression of this disease as well as identifying key VEGF regulatory points that could be targeted with chemotherapeutic agents.

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TRANSCRIPTIONAL RESPONSE OF VEGF IN BREAST CANCER CELLS

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Vascular endothelial growth factor (VEGF) expression is elevated in malignant cells, and clinical studies show a correlation between increased VEGF levels and development and prognosis of breast cancer. Relatively little is known about the transcriptional activation of VEGF in breast cancer, and the regulation of the gene may be distinct not only among different types but also within the same class of tumors. An understanding of how the gene is regulated in this disease could lead to improved treatment regiments: One can potentially design and synthesize novel expression vectors containing only the VEGF promoter elements required for high-level expression in breast cancer. Therapeutic genes would thus be specifically expressed in malignant but less so in normal cells.

Transient transfections of breast cancer cells with a full-length VEGF promoter construct (-1175 to + 50) driving the expression of the reporter gene firefly Luciferase have corroborated findings from other cell lines that VEGF expression is inducible by serum starvation irrespective of cell density. We have now introduced 5'-3' deletions every 100 base pairs (bp) into the full-length promoter beginning at -1010 and ending downstream at position -50 by either utilizing available restriction sites or using polymerase chain reaction (PCR) techniques. These 5' nested deletions will be transiently transfected into a panel of different breast cancer cell lines to identify the promoter elements that cause elevated VEGF breast cancer expression under various stimuli.

The relative shortness of such deletions will allow further characterization of transcriptionally active VEGF promoter fragments in breast cancer cells. Functional gene promoter elements can be cloned directly into promoterless cassettes to determine their activity in malignant and normal cells. Sequences of such elements will be scanned for putative transcription factor binding sites and specific binding to these sites analyzed by electrophoretic mobility shift assay (EMSA) using nuclear extracts. We anticipate that such studies will provide a) a better understanding of the mechanisms underlying the elevated activation of the VEGF gene in breast cancer, and b) a baseline for developing improved gene therapies for this disease.

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INHIBITION OF AP-1 TRANSCRIPTION FACTOR CAUSES BLOCKADE OF MULTIPLE-SIGNAL TRANSDUCTION PATHWAYS AND INHIBITS BREAST CANCER GROWTH

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AP-1 transcription factors play a critical role in signal transduction pathways in many cells. We have investigated the role of AP-1 in controlling proliferative signals in breast cells, and have previously shown that AP-1 complexes are activated by peptide and steroid growth factors in both normal and malignant breast cells. In this study, we investigated the role of AP-1 in transducing proliferative signals induced by peptide and steroid growth factors. We used MCF-7 clones that express a specific inhibitor of AP-1, a dominant-negative cJun mutant (TAM67), under the control of an inducible promoter to investigate the role of AP-1 in regulating breast cancer growth. In the presence of doxycycline (Dox), the AP-1 inhibitor was not expressed, and the MCF-7 clones proliferated normally in response to serum stimulation. However, when Dox was withdrawn, TAM67 was expressed, AP-1 activity was inhibited, and serum-induced proliferation was blocked. We next investigated whether the mitogenic response to specific growth factors also requires AP-1. MCF-7 Tet-Off-TAM67 cells were grown in the presence of increasing concentrations of IGF-1, EGF, heregulin-β, bFGF, or estrogen under un-induced and induced conditions. These studies showed that the AP-1 inhibitor completely blocked proliferation in response to the peptide growth factors (IGF-1, EGF, heregulin-β, and bFGF), and partially blocked the response to estrogen. To investigate the effect of AP-1 blockade on *in vivo* tumor growth, we injected the MCF-7 Tet-Off TAM67 cells into nude mice receiving doxycycline to suppress the expression of the AP-1 inhibitor. After the mice developed tumors, they were randomized to either continue to receive Dox or not. In mice not receiving Dox, the expression of TAM67 was induced, and tumor growth was inhibited, while the tumors in mice receiving Dox continued to grow. Analysis of the tumors from these mice showed that the expression of TAM67 caused reduced proliferation of the breast cancer cells without inducing apoptosis. These results demonstrate that AP-1 blockade suppresses mitogenic signals from multiple different peptide growth factors as well as estrogen, and inhibits the growth of MCF-7 breast cancer cells both *in vitro* and *in vivo*. These results suggest that novel agents specifically targeting AP-1 or its activating kinases could be promising agents for the treatment of breast cancer.

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REGULATION OF BRCA2 TRANSCRIPTION

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The BRCA2 breast cancer predisposition gene is a tumor suppressor gene that contributes to 20% of hereditary breast tumors and 2-3% of all breast tumors. The BRCA2 protein is involved in homologous recombination, base excision, nucleotide excision, and transcription coupled DNA damage repair. In addition BRCA2 regulates cell cycle control, cell growth, and chromosomal stability. Disruption of one or more of these functions by mutations leads to tumor development. Given the important role of this protein in regulating normal cellular function we proposed to examine whether exogeneous agents could alter BRCA2 expression and thereby contribute to breast cancer. Initially, deletion mapping of the promoter identified a USF1 binding domain as a critical element for basal activity of the promoter. Subsequently, USF1 was shown to co-operate with Ets factors to regulate activity. Further studies demonstrated that NFkB could induce promoter activity and increase BRCA2 expression levels. The effects of certain pharmacological agents on BRCA2 promoter activity have also been investigated. Estrogens, androgens, and xenobiotics such as benzopyrene have no effect on the promoter. However, certain DNA damaging agents such as adriamycin and mitomycin C substantially repress promoter activity in a p53 dependent manner. This effect is achieved by inhibition of USF1 binding to the promoter. Thus, in the presence of p53, BRCA2 expression is downregulated while in the absence of p53, BRCA2 has at least 10 fold higher expression during S and G2 phase of the cell cycle.

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CCAAT-DISPLACEMENT PROTEIN NEGATIVELY REGULATES MOUSE MAMMARY TUMOR VIRUS TRANSCRIPTION AND TUMORIGENESIS

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Mouse mammary tumor virus (MMTV) is a betaretrovirus that is transmitted from lactating mothers to pups through the milk. High levels of MMTV transcription in lactating mammary gland allows integration near cellular proto-oncogenes, leading to the induction of breast cancer. In contrast, MMTV expression is repressed in other tissues to prevent mutagenic integrations that may diminish mother-to-offspring transmission. A cellular homeodomain protein, CCAAT-displacement protein (CDP), represses basal and glucocorticoid-induced levels of MMTV transcription through multiple binding sites in the LTR. Interestingly, CDP-binding activity for the MMTV LTR decreases during mammary gland development, suggesting that reduced CDP binding may contribute to the elevated viral expression in lactating mammary gland. To investigate the contribution of CDP to MMTV replication in vivo, we engineered infectious MMTV proviruses with mutations that affected CDP binding in gel shift assays. Such mutant proviruses showed elevated expression, as determined by ribonuclease protection assays and Western blotting, compared to that of the wild type in transfected XC rat fibroblasts. Two of the proviral mutants (HP692 and HP838) that retain an intact superantigen (sag) open reading frame were inoculated into weanling BALB/c mice. Deletion of Sag-reactive T cells is a sensitive indicator of MMTV infection. Only one of the mutants (HP838) showed accelerated deletion of Sag-reactive T cells at multiple time points compared to that of wild-type inoculated mice, suggesting increased expression in B lymphoid cells. RNase protection experiments revealed that the levels of both mutants were elevated in virgin mammary glands of the injected animals. Mutation of CDP-binding sites also reduced the latency and increased the number of MMTV-induced mammary tumors per animal.

Since proviruses with single CDP-binding site mutations had a demonstrable phenotype, we reasoned that MMTV infection of CDP-null mice would also increase viral replication and tumorigenesis in the mammary gland. We infected newborn pups heterozygous for a deletion of one of the CDP DNA-binding domains (ΔCR1) with C3H MMTV by foster nursing on C3H/HeN Mtv+ mice. Preliminary results suggest that these (+/-) mice develop mammary tumors with shorter latencies compared to those of (+/+) littermates. These data suggest that CDP is a critical regulator of genes, such as those encoded by MMTV, that are transcribed in normal and neoplastic mammary tissue.

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ALTERNATIVE SPLICING OF THE NOVEL INSULATOR GENE BORIS IN BREAST CANCER

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Abnormal RNA splicing has been the focus of many laboratories studying breast cancer, and this alternative splicing is strongly linked to poor survival and metastatic disease independent of other risk factors. Alternative splicing appears to represent the aberrant expression of fetal isoforms of the affected genes. We have been investigating the role of this alternative splicing in breast cancer and its consequences for tumor cell growth. In the course of these studies, we have identified a novel insulator protein, termed BORIS (Brother of Regulator of Imprinted States), that is a paralogue of the known insulator protein CTCF involved in regulation of imprinting, as well as transcriptional regulation of c-myc and other genes. BORIS is located within the breast cancer amplicon on 20q13, and is overexpressed in some breast cancers. In order to determine whether alternative splicing of BORIS might be related to its normal or abnormal function in breast cancer, we determined the exon-intron structure and analyzed RT-PCR products for alternative splicing. We found that breast cancers frequently show alternative splicing between exon 1 and one of several alternative upstream exons. This alternative splicing is enhanced in tumors and leads to production of a "long" BORIS, with an alternative translation initiation site, adding at least 300 amino acids. These upstream sequences include multiple proline-rich motifs consistent with SH3 or WW-domain binding, and may be involved in localization and interaction with other proteins. These results are significant because they suggest a role for alternative splicing in breast cancer leading to epigenetic alterations such as loss of imprinting.

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HnRNP F AND H ARE POLYADENYLATION FACTORS IN PLASMA B CELLS: A POSSIBLE ROLE IN MULTIPLE MYELOMA

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Examination of the complexes formed on input pre-mRNA in nuclear extracts revealed large assemblages containing hnRNP H, H', and F but deficient in CstF-64 in memory B-cell extracts but not in plasma cells. Formation of these large complexes is dependent on the region downstream of the AAUAAA in pre-mRNA, suggesting that CstF-64 and the hnRNPs compete for a similar region. In vitro binding studies showed that hnRNP F and CstF 64 do compete to bind to pre-mRNA and this competition is influenced by Carboxy Terminal Domain (CTD) of RNA polymerase II. The effect of phosphorylated CTD on this competition will be investigated.

HnRNP F was shown to bind G-rich sequence downstream to GU-rich region to which CstF 64 binds. Interestingly, affinity of hnRNP F to pre-mRNA is higher than that of CstF 64. Overexpression of recombinant hnRNP F in plasma cells resulted in a decrease in the usage of promoter proximal strong polyA site of endogenous Ig heavy chain. This indicates that hnRNP F was able to show its inhibitory effect on polyadenylation in murine plasma cells.

A global analysis of gene expression is being done with hnRNP F and H transfected mammalian cells using microarray technology. We expect to identify other complex transcription units that may be controlled by these proteins, including genes differentially expressed in cancer cells.

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HOMEOBOX GENE EXPRESSION DURING WILD-TYPE OR P53 -/- MOUSE MAMMARY TUMORIGENESIS

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Homeobox-containing (Hox) genes encode transcription factors that control the patterning of the body plan and cell fate during mammalian embryo development. Recently, studies have indicated that Hox genes are also functional in adult animals. Aberrant expression of Hox genes has been found in many human cancers. To elucidate the potential roles of Hox genes during mammary tumorigenesis, we investigated the expression patterns of several Hox A-cluster genes using a mouse model system. In this system, a chemical carcinogen, DMBA, was used to induce mammary tumors in BALB/c mice that express wild type p53.

By analyzing the mRNA levels of several Hox A-cluster genes in wild type mammary tissues from different stages of tumorigenesis, we found that Hox a5 mRNA was expressed in normal mammary tissue, especially during pregnancy. A two-fold higher level of Hox a5 mRNA was observed in mammary tissue that had undergone hyperplasia. In some mammary tumors, the levels of Hox a5 mRNA were 4- to 7-fold higher than those in normal mammary tissue. Neither Hox a1 nor Hox a3 mRNA was detected in normal or hyperplastic tissue, but the levels of Hox a1 and Hox a3 mRNA were elevated in some of the breast tumors. To determine whether Hox A-cluster gene expression was mediated by retinoic acid receptor beta (RAR beta) gene expression in mammary tumorigenesis, we assessed the level of RAR beta mRNA. RAR beta mRNA was observed only at low levels in some tumors. When the expression of Hox a1, 3, 5 and RAR beta mRNA in mammary tissue lacking the p53 gene was measured, Hox a3 and Hox a5 were found to be expressed at significantly higher levels in the tumors than in the non-tumor tissues. RAR beta mRNA was not detected in non-tumor tissue or in the tumors which lacked the p53 gene. We further asked whether or not hormone generated from pituitary isografts (PIT), or the chemical carcinogen, DMBA, influenced the expression of these Hox A-cluster genes. Our results demonstrated that neither PIT nor DMBA treatment influenced Hox a1, a3, a5, or RAR beta mRNA expression.

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ESX TRANSFORMS MCF-12A HUMAN MAMMARY EPITHELIAL CELLS

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ESX is an epithelial-restricted member of a large family of transcription factors known as the Ets family. ESX expression is typically correlated with Her2/neu proto-oncogene amplification in highly-aggressive breast cancers and ESX is induced by Her2/neu in breast cell lines. In order to determine the role of ESX in mammary epithelial cell transformation and to determine whether it acts downstream of Her2/neu, we used the immortalized, but nontransformed MCF-12A human mammary epithelial cell line. Importantly, these cells do not express endogenous ESX protein. Transient transfection of MCF-12A cells with an HA-ESX expression vector resulted in increased colony formation compared to controls. Whereas, interference with ESX expression in transformed T47D cells greatly reduced colony formation of these cells, compared to controls. Stable transfection of MCF-12A mammary cells with the pCGN2-HA-ESX, pCGN2-HA-Ets-2 and pSVRas expression vectors revealed that stable expression of ESX induced EGF-independent proliferation, serum-independent MAPK phosphorylation and growth in soft agar. Additionally, stable ESX expression conferred increased cell adhesion, motility and invasion in two-dimensional and trans-well filter assays, and an epithelial to mesenchymal morphological transition. In three-dimensional cultures, parental and control (pCGN2) cells formed highly organized duct-like structures with evidence of cell polarity, ECM adhesion-dependent proliferation and cell survival, and lack of cellular invasion into surrounding matrix. Remarkably, the ESX stable cells formed solid, disorganized structures, with lack of cell polarity and loss of dependence on ECM adhesion for cell proliferation and survival. In addition, ESX cells invaded the surrounding matrix, indicative of a transformed and metastatic phenotype. The positive controls, pCGN2-HA-Ets-2 and pSVRas, also increased adhesion, motility and invasion; whereas the negative control cells lacked any evidence of the transformed phenotype. Finally, chromatin immunoprecipitation studies of MCF-12A cells infected with control or ESX-expressing adenoviruses showed that ESX binds to the endogenous Her2/neu promoter. Taken together, these data show that ESX expression alone confers a transformed and metastatic phenotype.

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NUCLEAR RECEPTOR COREPRESSOR REGULATION OF ESTROGEN RECEPTOR ANTAGONIST FUNCTION

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Defining the molecular mechanisms that integrate diverse signaling pathways at the level of gene transcription remains a central issue in biology. We have demonstrated that the nuclear receptor corepressor N-CoR is required for antagonist actions of 4-hydroxy tamoxifen (4-OHT). In the absence of N-CoR, 4-OHT acts as a full antagonist. We demonstrate that interleukin 1 (IL-1) causes nuclear export of a novel corepressor complex containing N-CoR, HDAC3 and TAB2, derepressing a specific subset of NF-kB-regulated genes, exemplified by tetraspanin KAI1 that regulates receptor function and specific nuclear receptors. Nuclear export of the N-CoR/TAB2/HDAC3 complex in response to IL-1 is temporally linked to selective recruitment of specific coactivator complexes. Intriguingly, while IL-1 treatment does not alter repressive events for the retinoic acid or thyroid hormone receptors, it results in conversion of estrogen receptor from antagonist to full agonist. 4-OHT-bound estrogen receptor binds to N-CoR/HDAC3/TAB2 complex, while liganded RAR does not. These data reveal that specific signaling pathways, in this case involving MEKK1 activation can mediate the actions of estrogen receptor antagonist, with clear implications for breast cancer therapy.

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INVESTIGATION OF ALTERATIONS IN PRE-MRNA SPLICING IN BREAST CANCER USING OLIGONUCLEOTIDE MICROARRAYS

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Growing evidence indicates that alternative or aberrant pre-mRNA splicing takes place during the development, progression, and maintenance of breast cancer. For example, splice variants of several genes linked to breast cancer are detected in breast cancer cell lines or tissues. These genes encode estrogen and prolactin receptors, Cyclin D1, BRCA-1, and BRCA-2 etc. The alterations in splicing patterns suggest deregulation of pre-mRNA splicing in mammary tumorigenesis. However, the regulatory sequences, the splicing factors, and the signal pathways involved in this deregulation have not been identified. Here we use microarray techniques to simultaneously assay splicing changes of a number of genes in breast cancer cell lines. The microarrays contain >400 oligonucleotides designed to detect alternatively spliced mRNAs from more than 60 human genes implicated in cancer progression and apoptosis. Poly(A)$^+$ RNA was isolated from human mammary epithelial cells and MCF7 breast cancer cells, labeled with Cy3 or Cy5, and hybridized to the glass oligonucleotide arrays. We detected interesting splicing changes in several genes related to apoptosis or proliferation in MCF7 cells as compared to normal cells. The utility of microarrays in splicing detection, the characterization of these splice variants, and the splicing alteration in breast cancer will be discussed.

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THE BRN-3B TRANSCRIPTION FACTOR REGULATES THE PROLIFERATION AND TUMORIGENIC ABILITY OF HUMAN MAMMARY CANCER CELLS

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The Brn-3b transcription factor has previously been shown by our laboratory to be over-expressed in human breast cancer tissue and to inversely correlate with expression of the anti-oncogene protein BRCA-1. We have now prepared cell lines derived from the human MCF-7 breast cancer cell line in which the expression of Brn-3b has been artificially enhanced or reduced (using an antisense approach). The cell lines over-expressing Brn-3b showed much more rapid growth and an enhanced saturation density compared to the control cells. In contrast, the cells with reduced expression of Brn-3b showed decreased growth rate and saturation density compared to the control cells. Similarly, the cells with enhanced Brn-3b levels show increased ability to grow in an anchorage-independent manner in soft agar whereas the cells with reduced Brn-3b expression show a decreased ability to form colonies in soft agar. Moreover, when these cell lines were inoculated into nude mice, cells over-expressing Brn-3b grew more rapidly than control cells and formed larger tumours. These experiments show for the first time that the Brn-3b transcription factor can regulate the growth of human breast cancer cells both in vitro and in vivo. In view of the over-expression of Brn-3b in human mammary tumours, this indicates that Brn-3b is likely to represent a key factor in regulating the growth of these cells and therefore to be a potential therapeutic target.

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REGULATION OF OSTEOPONTIN AND BONE SIALOPROTEIN EXPRESSION IN BREAST CANCER CELLS

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The majority of patients with advanced breast cancer will suffer from bone metastases, which account for significant morbidity and mortality. Components of the bone matrix include osteopontin (OPN) and bone sialoprotein (BSP), which share related protein structures. OPN and BSP have been a focus of special interest since ectopic expression of these proteins was demonstrated in breast cancer cells. Despite an experimental correlation with metastatic potential, there is little information available on how expression of OPN and BSP is initially induced in breast cells. This has been difficult to approach without an understanding of how these products are regulated in bone. From studies of basic mechanisms regulating proliferation and tissue specific gene expression in osteoblasts, we learned that OPN is induced specifically in response to increased phosphate levels, a normal part of bone development.

We are now expanding these studies to compare breast cancer cell lines that differ in OPN expression. MCF7 cells have normal levels of OPN, while MDA-MB-435 cells have elevated levels typical of metastasizing breast cancer cells. We have used RNA analysis to explore the hypothesis that increased phosphate levels, or a phenomenon we think may be related, i.e., oxidative stress, can induce OPN in breast cancer cells. Our results to date indicate that increased extracellular phosphate concentration is not sufficient to induce OPN expression in MCF7 cells as it is in the committed pre-osteoblast cell line MC3T3-E1. However, preliminary results suggest that increased OPN expression in breast cancer cells does correlate with elevated levels of the oxidative stress response transcription factor Nrf2. The identification of the molecular mechanisms that lead metastatic cancer cells to specifically seed the skeleton is a major challenge of cancer research. These studies have the potential to offer an improved basis for diagnosis and prognosis, and may also identify novel targets for chemotherapy.

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TRANSCRIPTIONAL REGULATION OF THE BRCA1 PROMOTER AND ITS ROLE IN SPORADIC BREAST CANCER

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It has become clear in recent years that decreased expression of the BRCA1 gene is frequently observed during the development of sporadic breast cancer and is correlated with tumor progression. This is consistent with a model where loss of BRCA1 function, either through mutations in the case of familial breast cancer or by decreased expression in the sporadic form, leads to tumor formation. We have been examining the transcriptional regulation of the BRCA1 gene promoter in an effort to identify proteins whose activity may be involved in the down regulation of this gene.

We have identified a site in the BRCA1 proximal promoter, referred to as the RIBS site, which is a major positive element in the human breast tumor line MCF-7. In contrast, this factor does not appear to be active in T-47D cells and reduced DNA binding activity to the RIBS site is observed in this line. We have identified the *ets* protein GABP ∀/∃ as the principle protein interacting with this site. All of the various GABP subunits appear to be expressed in both cell lines. We are currently investigating whether mutations in any of these subunits leads to their inactivation in T- 47D cells.

The BRCA1 promoter is part of a bi-directional transcription unit with the NBR2 gene lying just upstream. The RIBS element appears to act as a positive element for both promoters. We are currently examining the effect of mutations in the RIBS element for their influence on the differential utilization of both promoters. GABP∀/∃ binds as a tetramer containing two DNA binding subunits, so that its relative placement on the promoter may affect expression and its interaction with other transcription factors. This includes an adjacent CREB site, which is an apparent target for methylation and which we have determined is important for basel promoter activity but does not confer cAMP responsiveness.

We hope to identify genes which are responsible for the development of sporadic breast cancer. Understanding the regulation of the BRCA1 promoter may also allow for the development of novel methods of treatment.

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THE ROLE OF NUCLEAR RECEPTORS NOR-1 AND NUR77 DURING MAMMARY GLAND DEVELOPMENT

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The Nur subfamily of the Nuclear Receptor superfamily consists of three highly conserved transcription factors nor-1, nur77, and nurr1. All three proteins have the capacity to function redundantly by regulating the expression of overlapping target genes if coexpressed. While the physiological functions of these receptors are not well defined, studies to date have implicated this subfamily in the regulation of gene expression during proliferation, apoptosis, and tumorigenesis. The purpose of this study is to identify potential regulatory roles of the nur subfamily during murine mammary gland development.

Our first objective has been to analyze the spatial and temporal expression patterns of nor-1, nur77, and nurr1 during embryonic and postnatal mammary gland development. A transgenic lacZ reporter mouse and a series of ribonucleic protection assays have been utilized. We have found that nor-1 is specifically expressed in the mesenchyme of the embryonic mammary bud and the epithelial component of the gland during the later stages of post-lactation involution. The messenger RNA for nur77 has also been detected during involution, suggesting possible redundancy of function for this receptor. Nurr1 expression in the gland has not been detected. Our second objective has been to determine the consequence of ablation of any of or a combination of these receptors within the mammary gland. A detailed morphological and histological analysis of mammary gland development in available knockout mouse models has shown that selective ablation of either nor-1 or nur77 does not alter normal mammary gland development.

The distinct spatial and temporal expression pattern of the nur nuclear receptors in the gland suggests that they may regulate gene expression that controls proliferation, apoptosis, and/or stromal remodeling. Current studies are focused on analysis of mammary gland development upon ablation of both nor-1 and nur77. Understanding the regulation of gene expression during normal mammary gland development may provide insight into the networks that are disregulated during the process of tumorigenesis.

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INHIBITION OF GADD153/CHOP EXPRESSION BY NF-κB IN BREAST CANCER CELLS

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Exposure of mammalian cells to ultraviolet light, nutrient deprived culture media, hypoxia, environmental toxicants such as methyl mercury, methyl methanesulfonate, crocodilite asbestos or the agents that disrupt the function of endoplasmic reticulum (ER) leads to activation of the pro-apoptotic transcription factor GADD153/CHOP. Paradoxically, several of these agents also induce the anti-apoptotic transcription factor NF-kB. In this report, we demonstrate that NF-kB inhibits GADD153 activation in breast cancer cells exposed to nutrient deprived media, tunicamycin (which blocks protein folding in ER) or calcium ionopore (which depletes calcium stores in ER). Basal and calcium ionopore-induced GADD153 expression was more pronounced in fibroblasts obtained from mouse embryos lacking p65 subunit of NF-kB compared to fibroblasts from wild type littermate embryos. Moreover, p65-/- fibroblasts were killed more efficiently by calcium ionopore and tunicamycin but not hydrogen peroxide compared to wild type fibroblasts. We also show that parthenolide, a NF-kB inhibitor, sensitize breast cancer cells to tunicamycin. Transient transfection assay revealed that the p65 subunit but not the p50 subunit of NF-kB represses GADD153 promoter activity. These results establish a correlation between repression of pro-apoptotic genes by NF-kB and increased cell survival during ER stress. Additionally , by this mechanism, NF-kB may promote survival of cells that are damaged by environmental toxicants. A consequence of increased survival of damaged cells is malignant transformation. To test whether NF-kB promotes transformation of mammary epithelial cells damaged by environmental toxicants, we are currently generating mammary epithelial cell line MCF10A that overexpress the p65 subunit of NF-kB. In addition, we are generating cell lines that overexpress two subunits of a kinase that activates NF-kB constitutively. Malignant transformation of these upon DNA damage by environmental toxicants will be studied.

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TRANSCRIPTIONAL REPRESSION OF THE STEAROYL-COA DESATURASE GENE BY WILD-TYPE P53 IN HUMAN BREAST CANCER

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Stearoyl-CoA desaturase (SCD) is a central lipogenic enzyme catalyzing all reactions in the synthesis of monounsaturated fatty acids mainly oleate (C18:1) and palmitoleate (C16:1) which are the major monounsaturated fatty acids of membrane phospholipids, triglycerides, and cholesterol esters. SCD activity is increased in cancer and other situations of accelerated cell growth to provide more unsaturated fatty acids for membrane biogenesis, fluidity and increased cellular metabolism. p53 is a tumor suppressor gene vital for cell cycle regulation and its loss of function is associated with several human cancers. Previous studies on p53-repressed genes have implicated several genes involved in the cell cycle as targets of negative regulation by p53. However, few gene targets of p53-repression that are involved in lipid synthesis have been identified. This study has identified the stearoyl-CoA desaturase as a p53-repressed lipogenic gene in human breast cancer. DNA co-transfection, site directed mutagenesis and mobility shift studies in MCF-7 human breast cancer cells indicate that the p53 protein binds the SCD gene promoter at a site that overlaps the binding site for sterol regulatory binding protein-1a (SREBP-1a) and nuclear factor–Y (NF-Y) transcription factors both of which are involved in cholesterol and polyunsaturated fatty acid suppression of lipogenic gene expression. The mutated version of p53 protein does not bind the SCD promoter and fails to repress the transcription of the SCD gene. The identification of a common DNA promoter element of the wild type p53-, cholesterol- and PUFA-mediated repression of the SCD gene expression should aid in the elucidation of the interaction of p53 and dietary factors on the genetic regulation of lipogenesis in human breast cancer.

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CAP-DEPENDENT TRANSLATIONAL CONTROL OF TUMORIGENICITY AND CHEMORESISTANCE IN BREAST CANCER CELLS

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Tumorigenicity and escape from normal cell death mechanisms in cancer cells generally correlates with abnormaly high rates of protein synthesis. We previously discovered that in normal and oncogene-transformed rodent fibroblasts, tumorigenicity, apoptosis and cell chemoresistance are subjects to translational control. These experiments stimulated us to question whether translational control is relevant to the regulation of apoptosis in naturally occurring human breast cancer cells.

Our approach approved by USAMRMC was designed to test: (a) what are the molecular mechanisms maintaining an aberrantly activated translation initiation apparatus in human breast carcinoma cells; (b) whether constitutive activation of the translational machinery is sufficient to cause malignant conversion of non-transformed human mammary epithelial cells; and (c) whether targeted disruption of aberrant cap-dependent translation reverses the transformed phenotype and chemoresistance in breast cancer cells.

We demonstrate that cells of five breast carcinoma lines with diverse tumor-related gene alterations function in a translationally activated state as compared to non-transformed human mammary epithelial cells (HMEC). Introduction of a gene construct into non-transformed HMEC leading to constitutive expression of eIF4E confers the ability to form transformed foci in vitro. Accordingly, ectopic expression of wild type 4E-BP1 or its phosphorylation site mutants reduces the ability of breast cancer cells to form colonies in vitro and increases spontaneous or drug-induced apoptosis in a manner dependent on the potency of ectopically expressed 4E-BP1 to inhibit cap-dependent translation. Overexpressed wild type 4E-BP1 markedly diminishes tumorigenicity of MDA-MB-468 breast carcinoma cells injected into immunocompromized mice, whereas introduction of the a hypophosphorylated mutant of 4E-BP1 completely blocks tumor growth during ten days of observation. Together, these findings suggest that a hyperactivated cap-dependent translation apparatus is a characteristic trait of breast carcinogenesis, and an important mechanism by which cancer cells maintain their tumorigenicity, evade apoptosis, and acquire chemoresistance.

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BRCA2 AND THE VARIANT BRCA2A, LACKING THE TRANSACTIVATION DOMAIN FUNCTIONS AS A TRANSCRIPTIONAL COACTIVATOR OF P53, INTERACT WITH E2F, CYCLINS, AND CDKS

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BRCA1 and BRCA2 are tumor suppressor genes shown to be involved in 90% of familial breast cancers and also known to be involved in ovarian and prostate cancers. Both BRCA1 and BRCA2 gene products are regulated in a cell cycle-dependent manner and have potential transactivation function. Here, we show that BRCA2 undergoes differential splicing giving rise to a variant protein BRCA2a, lacking putative transcriptional activation domain. Both BRCA2a and BRCA2 are expressed at high levels in thymus and testis but moderate levels in mammary gland and prostate suggesting that BRCA2a and BRCA2 may have a role in the development and differentiation of these tissues. Here, we have studied the interaction of BRCA2 and p53. Our results demonstrate that BRCA2 interacts with p53. We have localized the domains of BRCA2 and p53 for protein-protein interaction. We also tested the effect of this interaction on p53 transactivation function. Our results demonstrate that BRCA2 cooperates with p53 to stimulate gene expression from a construct containing p53 responsive elements in a dose dependent manner. Thus, BRCA2 functions as a coactivator in p53-dependent gene expression. Since the expression of BRCA1 and BRCA2 gene products are coordinately regulated and their expression peaks at the G1/S boundary of the cell cycle, they may show similar functional properties in spite of the fact that they show no major sequence homology. Since BRCA1 interacts with E2F, cyclins and CDKs (which are known to regulate the cell cycle), we hypothesized that BRCA2 may be regulating the cell cycle by interacting with these proteins. To test this hypothesis, we have carried out GST- pull down assays to study the interaction of BRCA2 with E2F, cyclins and CDKs. Our results demonstrate that both BRCA2 and BRCA2a associate with transcriptional factor E2F, cyclins and cyclin dependent kinases (CDKs). We have localized the domains of BRCA2 that is responsible for these protein-protein interactions. Patients with mutations in these domains may have impaired binding of BRCA2 to E2F, cyclins/CDKs, resulting in a loss of the negative regulation of cell cycle/cell proliferation and predisposition to breast cancers.

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IDENTIFICATION OF ESTROGEN-RESPONSIVE GENES AND THEIR ROLE IN BREAST CANCER

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Estrogen plays an important role in proliferation and progression of breast cancer. It modulates expression of specific genes in neoplastic as well as in normal breast cells through a ligand-activated transcription factor, the estrogen receptor (ER). The aim of this study was to identify genes that are differentially expressed in response to estradiol treatment in hormone-responsive breast carcinoma cells.

Using suppression subtractive hybridization, a set of 14 estrogen-responsive genes were isolated that was differentially overexpressed in MCF-7 cells stimulated by beta-estradiol as compared with unstimulated cells. Tamoxifen, a non-steroidal anti-estrogenic agent, repressed the expression of all these genes. PDZK1 and a novel gene - GREB1, were confirmed to be early responders to estrogen and demonstrated a significant correlation to the ER phenotype in a panel of ER positive and negative breast carcinoma cell lines. Treatment with cycloheximide indicated that ER directly controlled GREB1 expression, whereas PDZK1 appeared to be a secondary target. Studies in primary breast cancers showed that PDZK1 and GREB1 were overexpressed in ER-positive breast cancers as compared with ER-negative breast cancers by 19-fold and 3.5-fold respectively. Due to the strong correlation of GREBI and PDZKI expression to estrogen related responses, these genes were chosen as markers to test drug responses in MCF7 cells and in primary breast tumors. Preliminary results have been promising and indicate the feasibility of detecting qualitative and semiquantitative differences in the expression of target genes- GREB1 and PDZK1- upon drug treatment.

The pattern of expression of GREB1 and PDZK1 suggests an important role for these proteins in physiological response of tumors to estrogen. There may be a trend that tumors, which exhibit a tamoxifen-induced alteration in the target gene expression in vitro, are more likely to respond to tamoxifen therapy whereas those, which do not, may be less likely to respond to tamoxifen treatment in a clinical setting. GREB1 and PDZK1 may thus serve as useful markers and studying the alteration in the expression of these marker genes may be used as a tool to predict a patient's response to drug treatment.

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THE EFFECT OF ESTRADIOL AND HISTONE DEACETYLASE INHIBITORS ON THE ACETYLATION OF HISTONES ALONG THE ESTROGEN-RESPONSIVE pS2 GENE

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Histones are subject to a variety of post-translational modifications, the most studied being acetylation of the N terminal lysine residues. Acetylation is thought to be a dynamic event mediated by the actions of histone acetyltransferases and deacetylases. The treatment of estrogen-responsive MCF-7 human breast cancer cells with estradiol causes the recruitment of histone acetyltransferases to the promoter region of estrogen-responsive genes such as pS2. Whether estradiol affects the distribution of histone deacetylases along this region remains to be determined. Furthermore, the effect of estradiol on the acetylation of histones along the coding regions of estrogen-responsive genes is unknown. MCF-7 human breast cancer cells were treated with or without 10 nM estradiol for 60 min and the DNA sequences associated with hyperacetylated histones H3 and H4, as well as acetylated lysine were isolated by chromatin immunoprecipitation. Analysis of the immunoprecipitated DNA by PCR showed an increase in acetylated H3, H4 and lysine along the pS2 promoter, exon 2 and exon 3 regions after treatment with 10 nM estradiol for 60 min. Treatment of MCF-7 cells with 10 mM sodium butyrate for 2 h, a histone deacetylase inhibitor, did not significantly alter the levels of acetylated histones along these pS2 gene regions in the absence of estradiol. However, addition of 10 nM estradiol to butyrate-treated MCF-7 cells for the second hour of butyrate treatment increased the level of acetylated H3, H4 and lysine at the pS2 promoter, exon 2 and exon 3. These results suggest that histone acetyltransferases are recruited to the promoter and coding region of the pS2 gene in the presence of estradiol, and that this acetylation is a dynamic event mediated by both histone acetyltransferases and histone deacetylases.

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FLUORESCENT REPORTERS TO RECAPITULATE IN VIVO ALTERNATIVE SPLICING REGULATION

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Fibroblast growth factor receptor 2 (FGF-R2) alternative splicing occurs in a highly tissue restrictive fashion. The mutually exclusive inclusion of exon IIIb or IIIc results in receptors with different ligand binding specificities. The general profile of FGF-R2 splicing demonstrates that the IIIb exon is predominantly included in epithelial cells while the IIIc exon is included in mesenchymal tissues. In recent elegant experiments, either the IIIb or IIIc exon has been knocked out in mice. FGF-R2-IIIb$^{-/-}$ mice survive to term but show severe dysgenesis and agenesis of many internal organs. Specific knockout of FGF-R2-IIIc results in an embryonic lethality, however, hemizygous knockouts demonstrate many of the physical abnormalities associated with Apert's and Pfeiffer's syndromes. Our lab has been studying the mechanisms of FGF-R2 alternative splicing using two rat cell lines that splice the endogenous FGF-R2 either to the IIIb exon or the IIIc exon, exclusively. Using this system we have identified several *cis* regulatory elements. In AT3 cells, which include IIIc, we have found that two Intronic Splicing Silencers (ISS) flank the IIIb exon and are each required for its repression. In DT3 cells, two cell-type specific *cis* elements, termed the Intronic Activating Sequence 2 (IAS2) and the Intronic Splicing Activator and Repressor (ISAR) have been identified to overwhelm the repressive effect of the flanking ISS. These two elements can form a putative secondary structure that we believe physically interferes with the activity of downstream ISS due to its proximity with IAS2. Our research on the mechanism of FGF-R2 alternative splicing has focused primarily on *in vitro* systems and tissue culture cell lines. We are interested in studying the physiological relevance of these mechanisms within the living animal to gain insight both on the developmental regulation of FGF-R2 alternative splicing as well as the regulation within the adult mouse.

We are developing a versatile set of fluorescent reporters using both GFP and RFP. Within the GFP reading frame we have introduced FGF-R2 exon IIIb as well as its regulatory *cis* elements. Inclusion of the IIIb exon within GFP cDNA results in an insertion, which disrupts fluorescence; only cells that skip the exon will fluoresce. Using this reporter, we have been able to demonstrate striking cell-type specific GFP fluorescence. We are also introducing the IIIc exon as well as its *cis* element regulatory sequences into the RFP reading frame. We are performing experiments aimed at illustrating cell-type specific fluorescence of RFP. These reporters, when used in concert, act as complementary measurements of activity of the splicing factors responsible for tissue-specific FGF-R2 alternative splicing. The long-term goal of these reporters is to elucidate the alternative splicing changes that occur in tumors in situ. Many genes undergo significant alternative splicing changes as breast cancer tumors progress; CD44 is a notable example. The GFP reporter has been designed in such a way as to accommodate the study of virtually any alternatively spliced exon. The potential uses of this reporter in breast cancer research will be discussed.

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CHARACTERIZATION OF p270 (*SMARCF1*) AND OTHER SWI/SNF PROTEINS IN HUMAN BREAST AND OTHER CANCER CELL LINES

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p270 was identified in our lab during studies of proteins, that affect gene expression during growth and differentiation. Cloning of p270 revealed that p270 is an integral member of human SWI/SNF complexes. These complexes can regulate gene expression by remodeling chromatin structure. p270 also belongs to a family of proteins containing a newly identified DNA binding domain, termed ARID. Besides the ARID domain, p270 has multiple nuclear hormone receptor binding motifs. A role for the SWI/SNF complex in human breast cancer has been suggested by the fact that the complex can modulate estrogen receptor and glucocorticoid receptor transcriptional activity. The multiple nuclear hormone binding motifs found in p270 suggest that p270 may mediate this activity via its receptor binding motifs as well as its DNA binding affinity. Components of SWI/SNF have been found frequently missing in tumor cell lines of different origins. In order to characterize the expression of p270 and other SWI/SNF proteins, we have developed mouse monoclonal antibodies against p270 as well as two other core SWI/SNF components, BRG1 and BAF155. With the aid of these antibodies and a commercially available antibody against hBRM, we screened 5 breast cancer cell lines plus 16 other tumor cell lines of different origins for expression of these SWI/SNF components. Consistent with the findings of others we observe that expression of SWI/SNF complex components is frequently lost in tumor cell lines. p270 likewise appears to be targeted during tumorigenesis. Examination of p270 expression at the RNA level in two different p270-deficient lines (T47D breast carcinoma cell line and C33A cervical carcinoma cell line) indicates that different mechanisms can underlie the loss of p270. In contrast to the breast, prostate and other carcinoma lines, in approximately half of which expression of at least one of the SWI/SNF proteins examined here was found to be abnormal, all six osteosarcoma cell lines we screened have normal levels of p270, BRG1, hBRM and BAF155 expression. These studies are likely to identify new markers for cancer diagnosis and prognosis.

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DNA-BINDING PROPERTIES OF P270, A MEMBER OF THE ARID FAMILY OF DNA-BINDING PROTEINS IMPLICATED IN NUCLEAR HORMONE RECEPTOR SIGNALING

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P270 is an integral member of the human SWI/SNF complexes, ATP-dependent chromatin remodeling complexes that contribute to gene regulation in response to hormone signaling. p270 has multiple LXXLL motifs, which are known to be involved in nuclear hormone receptor signaling, and a highly conserved DNA-binding domain termed ARID (A-T Rich Interaction Domain). The ARID family consists of 13 mammalian proteins several of which have been implicated in tumorigenesis (reviewed in Wilsker et al., 2002). Contrary to the name, we have found that p270 does not bind DNA in a sequence specific manner, implying it has a more global role within the SWI/SNF complex. Presumably, nuclear hormone receptor signals combine with the DNA binding activity of p270 to affect the activity of the SWI/SNF complex.

I have used specific monoclonal antibodies and DNA affinity chromatography columns to assess the DNA binding activity of endogenous p270 in hormone responsive cells. This analysis reveals that p270 has an unusually high affinity for single-stranded DNA. This affinity is comparable to another well-defined single-stranded DNA-binding protein, adenoviral 72K DBP (DNA-Binding Protein). This property is dependent on the integrity of the ARID sequence and is specific to the p270 ARID. Sequence specific ARID proteins do not share this property. We have concluded that p270 has an unusual DNA-binding activity which may be key to its role within hormone-responsive complexes. A full understanding of the relationship between nuclear hormone receptor interaction and p270 DNA binding activity will offer new insight into the mechanisms linking hormone signals and gene expression in breast cells, and may offer new targets for diagnosis and therapy.

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REGULATION OF SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) COACTIVATOR ACTIVITY BY IκB KINASE

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In the past few years, many nuclear receptor (NR) coactivators have been identified and shown to be an integral part of receptor action. The most studied of these coactivators are members of the steroid receptor coactivator (SRC) family, SRC-1, TIF2/GRIP1/SRC-2 and pCIP/ACTR/AIB-1/RAC-3/TRAM-1/SRC-3. The functions of these coactivators are important as loss of SRC-1 has been shown to cause defects in mammary gland development and overexpression of SRC-3 has been found in both primary human breast cancer samples and cancer cell lines.

To better understand the function of SRC family of coactivators, we purified these coactivator complexes by antibody affinity columns, and subsequently identified the associated proteins by mass spectrometry.

Surprisingly, we found association of SRC-3 but not SRC-1 with the IκB kinase (IKK). IKK is known to be responsible for the degradation of IκB and the subsequent activation of NF-κB. We demonstrated that SRC-3 is able to enhance NF-κB-mediated gene expression in concert with IKK. In addition, we showed that SRC-3 is phosphorylated by the IKK complex *in vitro*. Furthermore, elevated SRC-3 phosphorylation *in vivo* and translocation of SRC-3 from cytoplasm to nucleus in response to TNF-α occurred in cells, suggesting control of subcellular localization of SRC-3 by phosphorylation.

Taken together, our results not only reveal the IKK-mediated phosphorylation of SRC-3 as a regulated event that plays an important role, but also substantiate the role of SRC-3 in multiple signaling pathways. More importantly, as NF-κB plays a key role in cell survival, our results further suggest a possible role for SRC-3 in tumorigenesis through collaboration with NF-κB.

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AN MDM2-HDAC1 COMPLEX CONTROLS P53 ACETYLATION AND STABILITY BY A POTENTIAL COUPLING OF P53 ACETYLATION AND UBIQUITINATION

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The tumor suppressor p53 is stabilized and activated in response to cellular stress through posttranslational modifications including acetylation. Acetylation of p53 by p300/CBP is inhibited by its negative regulator, MDM2. Here we show that MDM2 promotes p53 deacetylation by recruiting a complex containing HDAC1. The HDAC1 complex binds MDM2 in a p53-independent manner and efficiently deacetylates p53 in vivo at all known acetylated lysines. Ectopic expression of a dominant negative HDAC1 mutant restores p53 acetylation in the presence of MDM2, whereas wild-type HDAC1 and MDM2 deacetylate p53 synergistically. Fibroblasts that over-express a dominant negative HDAC1 mutant display enhanced DNA damage-induced p53 acetylation, increased levels of p53, and a more pronounced induction of p21. These results indicate that acetylation promotes p53 stability and function nevertheless acetylation is not essential for p53-mediated transcription. As the acetylated p53 lysine residues overlap with those that are ubiquitinated, our results suggest that the major function of p53 acetylation is to promote p53 stability by preventing MDM2-dependent ubiquitination, while recruitment of HDAC1 by MDM2 promotes p53 degradation by removing these acetyl groups.

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TRANSCRIPTIONAL REGULATION OF HER-2/NEU EXPRESSION BY DNA MINOR-GROOVE-BINDING SMALL MOLECULES AND RECOMBINANT REPRESSOR PROTEINS

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Overexpression of the HER-2/neu oncogene is associated with aggressive tumor formation, characterized with shorter relapse times and higher chemo-resistance to the commonly used chemotherapeutic agents. In an effort to transcriptionally downregulate the HER-2/neu expression, we have targeted the gene promoter's TATA box with DNA minor groove binding hairpin polyamides and the AP-2 binding site with recombinant proteins consisting of a DNA binding domain fused to a repressor motif. The DNA binding specificity of the hairpin polyamide ImPyPyPy-gamma-PyPyPyPy-beta-Dp for the sequence A/T GTATA was determined by the combinatorial method REPSA and confirmed by DNase I footprinting. EMSA studies show that the hairpin polyamide could competitively replace TBP binding to the HER-2/neu TATA box at nano molar concentrations. Expression vectors of the recombinant repressor proteins were transfected into breast cancer cells in presence or absence of the hairpin polyamide or doxorubicin. The recombinant proteins and the hairpin polyamide showed synergistic downregulation of HER-2/neu expression from a promoter reporter construct and the endogenenous HER-2/neu gene. The hairpin polyamide and the recombinant repressor proteins were able to synergistically inhibit proliferation of the HER-2/neu overexpressing SKBR-3 cells and also enhance chemosensitivity of the tumor cells to doxorubicin. This analysis indicates that transcriptional regulation of HER-2/neu by the combination of hairpin polyamide and the recombinant repressor proteins could lead to a promising therapeutic approach for reducing the malignant phenotype of HER-2/neu overexpressing cancers.

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MECHANISM OF RNA INTERFERENCE

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My laboratory continues to pursue two parallel interests. First, we strive to understand the process of neoplastic transformation using cultured normal and cancer cells as models. Second, we investigate the mechanistic basis of dsRNA-induced gene silencing (RNAi) in experimental systems ranging from fungi to mammals. For several years, we have been working toward synthesizing these two interests for the creation of technologies that permit the use of genetic approaches toward the identification of novel anticancer targets. Through our mechanistic studies of RNAi, we have made substantial progress toward the creation of tools that will permit this goal to be achieved.

This year, we have made considerable progress in deciphering the mechanism of RNAi with the cloning of several proteins that are key to this gene silencing process. Among these was Dicer, the enzyme that initiates RNAi by processing dsRNAs into discrete ~22 nt siRNAs (small interfering RNAs). Genetic studies of Dicer in *C. elegans*, in collaboration with Ronald Plasterk of the Hubrecht Laboratories in Holland, revealed that Dicer had a role in regulating endogenous genes that control developmental timing. This prompted a paradigm shift in the field that now recognizes that the RNAi machinery may have a broad role in regulating cellular gene expression. An understanding of the enzymology of Dicer also led to two key discoveries, which may ultimately enable a fusion of our two core interests. Other laboratories (Elbashir and colleagues at the Max Plank Institute in Germany) demonstrated that chemically synthesized Dicer products are effective inducers of gene silencing in mammalian cells. The use of siRNAs is rapidly becoming a standard methodology for probing gene function in mammals. We have extended their seminal discovery through the development of cost-effective, high-throughput methodologies for constructing siRNAs and are now beginning a project to assess loss-of-function phenotypes on a whole-genome scale. We have recently found that the endogenous triggers of the RNAi pathway, which were linked to RNAi via genetic analyses of Dicer, can be retargeted to suppress a gene of interest. These shRNAs (small hairpin RNAs) can be stably expressed in vivo to create continuous cell lines, and perhaps even animals, in which RNAi is used to create a gene knock-out (or more precisely knock-down).

One goal of these studies is to develop tools, which will allow us to dissect the mechanisms of neoplastic transformation through the use of genetics in cell culture. A prerequisite to such an approach is the development of genetically defined human cancer models. Over the past two years, we have identified a combination of viral and cellular oncogenes that can transform normal human cells into cancer cells in vitro. We can now combine these defined models with RNAi-based loss-of-function approaches to search for genes that are essential to the survival and proliferation of cancer cells. Through such approaches, we hope to define new potential targets for selective anti-cancer therapies.

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COMBINATION THERAPY EMPLOYING RETINOIDS AND CHROMATIN REMODELING AGENTS IN HUMAN BREAST CARCINOMAS

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A strong inverse relationship between vitamin A and cancer development has been established by numerous investigations over the last few decades. Vitamin A deficiency in experimental animals has been associated with a higher incidence of cancer and with increased susceptibility to chemical carcinogens. All-trans-retinoic acid (RA), an active form of vitamin A, has been used in both chemotherapy and chemoprevention of human cancers. To understand the actions of RA, we have screened a RA-treated-F9 teratocarcinoma cell cDNA library by differential hybridization for genes that are regulated by RA. We isolated a 1,745-nucleotide cDNA for a gene encoding a transcription factor, Rex-1 (for reduced expression), which is expressed at high levels in F9 stem cells. Its expression is rapidly reduced upon RA treatment of F9 cells. In embryonic stem (ES) cells the expression of Rex-1 is greatly reduced upon LIF removal. Furthermore, Rex-1 has been used as a differentiation marker for many types of stem cells. Since the Rex-1 gene encodes a transcription factor, we decided to search for Rex-1 target genes in order to understand the functions of Rex-1. We had previously made an F9 Rex-1-/- cell line by homologous recombination. We employed Affymetrix microarrays, using total RNA isolated from F9 WT and F9 Rex-1-/- cells, treated +/- RA, to search for Rex-1 target genes. Many putative Rex-1 target genes were identified, including transcription regulators, apoptosis factors, tumor suppressors, growth factors, signaling factors, and hormone receptors, which play important roles in cancer development. The validity of these targets was confirmed by Northern analyses. We are now carrying out detailed studies of some of these Rex-1 target genes in various cell systems in order to understand the mechanism of action of Rex-1 in RA induced stem cell differentiation. Understanding the mechanism by which RA causes cell differentiation will contribute to the understanding of various types of cancer, and should lead to improved cancer therapies.

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